The Plant Journal (1994) 6(3), 321–338

LAT52 protein is essential for tomato pollen development: pollen expressing antisense *LAT52* RNA hydrates and germinates abnormally and cannot achieve fertilization

Jorge Muschietti, Lori Dircks, Guy Vancanneyt† and Sheila McCormick*
Plant Gene Expression Center, USDA/ARS-UC-Berkeley, 800 Buchanan St, Albany, CA 94710, USA

Summary

The *LAT52* gene of tomato is expressed in a pollen-specific manner. It is shown that *LAT52* encodes a heat-stable, glycosylated protein that traverses the secretory pathway when expressed in a baculovirus expression system. The LAT52 protein shows some similarity with Kunitz trypsin inhibitors and with pollen proteins from maize, rice and olive, but the biological function of these pollen proteins is unknown. To test whether the LAT52 protein plays an important role during pollen development, tomato plants were transformed with an antisense *LAT52* gene driven by the *LAT52* promoter. Because the *LAT52* gene is expressed gametophytically, only 50% of the pollen of the primary transformants would be expected to express the antisense construct. Self-progeny of 19 of the primary transformants showed the predicted 3:1 segregation for a single locus insertion of the linked kanamycin-resistance gene. However, the self-progeny of the other 32 primary transformants showed a 1:1 segregation pattern and could not transmit the linked kanamycin-resistance gene through the male. A subset of these 1:1 segregation class plants was examined in detail. The pollen showed lower levels of *LAT52* mRNA and LAT52 protein when compared with wild-type. *In vitro*, approximately 50% of the pollen grains appear to hydrate abnormally; this anomaly is not present when the same pollen grains are incubated in a medium with higher water potential. *In vivo* pollination experiments showed that the growth of around 50% of the pollen tubes is arrested in the style. The 3:1 segregation class plants showed no significant differences from untransformed control plants. Taken together, the results show a direct correlation between the reduced expression of LAT52 protein and abnormal pollen function, and suggest that the LAT52 protein plays a role in pollen hydration and/or pollen germination.

Received 13 December 1993; revised 8 April 1994; accepted 18 May 1994.
*For correspondence (fax +1 510 559 5678).
†Present address: Centro Nacional de Biotecnología, Universidad Autónoma de Madrid, Campus de Cantoblanco, E-28049, Madrid, Spain.

Introduction

Pollen development in flowering plants involves a complex series of events that begins with pollen mother cell meiosis and culminates with the entry of the male gametophyte into the embryo sac and gamete discharge (reviewed in McCormick, 1993). After meiosis, each microspore undergoes an asymmetric mitosis to produce a bicellular pollen grain composed of a large vegetative cell and a small generative cell. The synthesis of the pollen cell wall begins after meiosis, and at maturity is composed of two layers, the outer (exine) and the inner (intine). The exine shows species-specific ornamentation and is composed largely of a very resistant substance (sporopollenin), as well as sporophytically derived proteins that are deposited by the tapetal layer of the anther. The intine is composed of a cellulose-like fibrillar component and pectin, as well as gametophytically derived proteins. Mature pollen is somewhat dessicated, and hydration occurs via water uptake through the apertures, the sites of pollen tube emergence. Pollen germination and pollen-tube growth involve controlled and interactive biochemical, physiological and structural changes (reviewed in Mascarenhas, 1993), including mobilization of reserves to drive the rapid wall synthesis and the generation of calcium gradients necessary for polar growth.

Many pollen-specific genes have been isolated (reviewed in McCormick, 1991b) and the proteins encoded by them are surmised to play roles during pollen germination or pollen-tube growth, but none as yet has a proven function in these processes. The cellular location of these proteins and their timing of expression can provide some clues to function. For example, Brown and Crouch (1990) used tissue printing to show that the polygalacturonase-like P2 protein was localized to the pollen-tube, consistent with its presumed role in pollen tube wall synthesis, but the technique was not sensitive enough to discern whether the P2 protein was located in the pollen-tube wall. Dubald *et al.* (1993) used immuno-

localization to demonstrate that a maize polygalacturonase was cytosolic before pollen germination, and in the pollen-tube wall after germination. Mutational analysis can also be used to address the question of protein function during pollen development. Mutants that are deficient in a particular protein provide evidence that the protein is needed in the process that is defective in the mutant. For example, an adenine phosphoribosyl transferase mutant (*aprt*-) of *Arabidopsis* was shown to have defective pollen (Regan and Moffatt, 1990), suggesting a role for the nucleotide salvage pathway during pollen development. White pollen (chalcone synthase) mutants are male sterile unless pollen is supplied exogenously with flavonols, indicating a requirement for flavonoids during pollen germination (Mo *et al.*, 1992). An *Arabidopsis* mutant (*pop1*) that is defective in wax formation on the pollen coat is male sterile under conditions of low humidity (Preuss *et al.*, 1993), suggesting that lipids on the pollen coat are important for pollen hydration. Antisense technology provides a different approach to deduce the function of a known protein in some developmental process. If a pollen-expressed protein plays a necessary role during pollen development, an altered pollen phenotype should be found in the antisense transgenic plants.

The pollen-expressed *LAT52* gene encodes an 18 kDa protein that is cysteine rich and has an N-terminal putative secretory signal and a potential N-linked glycosylation site (Twell *et al.*, 1989b). We previously noted (Twell *et al.*, 1989b) that the LAT52 protein shows 32% amino acid identity to the Zm13 pollen-specific protein from maize (Hanson *et al.*, 1989). Recently a pollen-specific protein from rice (PS1) was also shown to be 36% similar at the amino acid level to LAT52 (Zou *et al.*, 1994), as was the major pollen allergen (Ole e I) of olive (Villalba *et al.*, 1993).

In this paper we biochemically characterize the LAT52 protein and use a baculovirus expression system towards determining its cellular location. To test whether the LAT52 protein is important for pollen development, we generated transgenic plants harboring an antisense copy of the *LAT52* cDNA. Using a combination of genetic, molecular and histochemical analyses, we show that pollen grains that are expressing an antisense version of the *LAT52* gene are different from wild-type pollen in their hydration properties, and that they have abnormal pollen-tube growth and consequently cannot achieve fertilization, indicating a critical role for the LAT52 protein during these processes.

Results

We used the construct shown in Figure 1 to generate transgenic tomato plants. The *LAT52* cDNA was inserted in antisense orientation between a 3 kb *LAT52* promoter and the 3' end of the *NOS* gene in pLAT52 (Twell *et al.*, 1991). This fragment was then excised by *Xba*I/*Bam*HI digestion and subcloned into pBin19 (Bevan, 1984), which carries a kanamycin-resistance gene as a selectable marker for transformation (see Experimental procedures). Tomato (cv. VF36) cotyledons were transformed (McCormick, 1991a) as described. In primary transformants (R_0 generation) with a single locus insertion, only 50% of the pollen would be expected to express an antisense copy of the *LAT52* gene, because the *LAT52* promoter used to drive antisense expression is gametophytically expressed (Twell *et al.*, 1990) in the vegetative cell of the pollen grain (Twell, 1992).

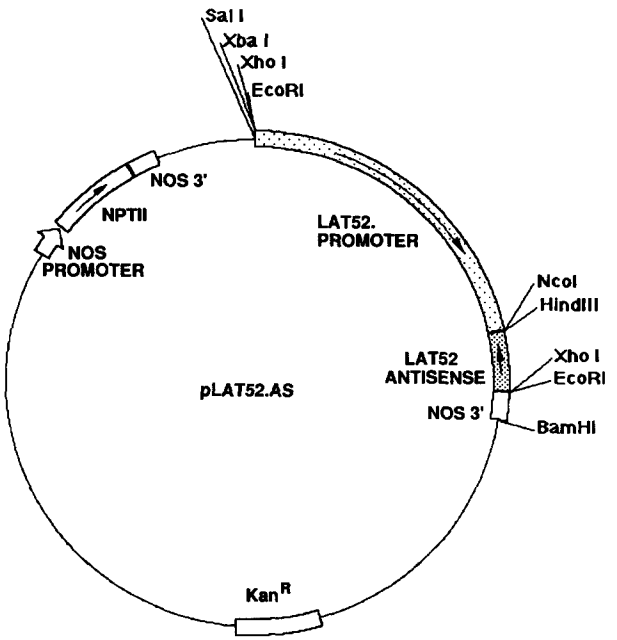

Figure 1. Structure of pLAT52.AS.
Gene fragments are drawn to scale.

Genetic analysis

Preliminary experiments with a small number of transgenic plants transformed with a *LAT52* promoter–*LAT52* antisense construct (McCormick *et al.*, 1991a) suggested that the antisense transgene was not transmitted in the expected Mendelian ratio, but these plants were not molecularly analyzed. We therefore transformed and regenerated 51 additional primary transformants carrying the *LAT52* antisense construct. These plants were allowed to self-pollinate, and in some cases (see Table 1) were outcrossed to untransformed tomato. In order to score for transmission of the antisense gene, transmission of the linked kanamycin-resistance gene was tested by germinating the R_1 seeds on kanamycin medium. Of the 51

primary transformants, 19 gave an apparent 3:1 segregation for kanamycin resistance, as expected for a single locus, while 32 of the plants showed an apparent 1:1 segregation for kanamycin resistance. A 1:1 ratio from a self-pollination implies that the resistance gene is not transmitted normally, but cannot distinguish whether the abnormal segregation is through the male or female. Backcrosses showed that the 1:1 class plants could not transmit the kanamcyin resistance through the male. These results are consistent with the notion that 50% of the pollen from the 1:1 class of R_0 plants is non-functional, specifically those pollen grains that carry the antisense *LAT52* gene and the linked kanamycin-resistance gene.

The flow-charts in Figure 2 illustrate the genetic analysis procedures. For example, self-crossing of primary transformant (R_0 generation) plant 6 gave 430 seeds, which when planted on kanamycin medium gave an approximate 3:1 ratio (chi-square value of 1.5). Ten kan^R seedlings from these 311 kan^R seedlings were transplanted to soil and designated R_1 generation plants. Self-crossing of two of them (plants B and H) demonstrated that plant B was a heterozygote for the kanamycin-resistance marker (chi-square value of 0.96), while the progeny of plant H were all kanamycin resistant, indicating that plant H was homozygous for the linked antisense construct. Self-crossing of plant 29 gave 333 seeds, which when planted on kanamycin medium gave an apparent 1:1 ratio (chi-square value of 2.7). Eleven kan^R seedlings were transplanted to soil and designated R_1 generation plants. Self-crossing of two of them (plants A and F) demonstrated that each showed a 1:1 segregation pattern for the linked antisense construct (chi-square values of 0.28 and 0.14).

Chi-square tests on all the R_1 segregation data in Table 1 revealed that plants 1–14 from the 3:1 segregation class showed segregation values (considering $P < 0.01$) that justified their inclusion in the 3:1 class. However, chi-square values for the R_1 self-cross progeny of plants 15–19 indicated that they exceeded the $P < 0.01$ significance level. None the less, for three of these plants (plants 16, 18 and 19) the backcross ratios (chi-square level, $P < 0.01$) support that they should be included in the 3:1 segregation class. Plants 15 and 17 are grouped with the 3:1 segregation class because the pollen phenotype is normal and because the R_2 segregation ratios more closely approximate a 3:1 ratio than a 1:1 ratio.

The R_1 segregation ratios for plants 20–42 justified their inclusion in the 1:1 segregation class (chi-square level, $P < 0.01$). The R_1 segregation values for plants 43–51 exceeded the $P < 0.01$ significance level. However, the chi-square values for R_2 progeny of plants 43, 44, 46 and 47 are significant ($P < 0.01$), and indicate that these plants should be grouped with the 1:1 segregation class. We believe that plants 45, 48, 49, 50 and 51 should also be grouped with this class, based on pollen phenotype and RNA and protein analyses (see below).

In addition to following the kanamycin-resistance marker as a measure of the presence of the antisense construct, genomic Southern analysis with kanamycin-resistant R_2 progeny of 24 of the 51 primary transformants was used to confirm directly the presence of the antisense construct and to assess its copy number. An example Southern blot is shown in Figure 3 and the results are tabulated in Table 1. All plants show a hybridizing band at 15 kb which represents the endogenous copy of the *LAT52* gene. The *Hin*dIII digest cuts genomic DNA external to the cDNA fragment used as the hybridization probe, so additional hybridizing fragments identify copies of the antisense construct. Plants 38 and 49 had a single additional band, while plant 9 showed two copies of the construct. We found no direct correlation between the copy number of the construct and the pollen phenotypes. For example, several of the 3:1 segregation class plants have more than one copy (presumably at one genetic locus), while some of the 1:1 class plants with the most extreme phenotype (plants 20, 38 and 49) carry only one copy of the transgene.

Kanamycin-resistant R_1 progeny from the 1:1 primary transformants exhibited the same phenotype as the parents (i.e. 1:1 plants again gave 1:1 ratios in the R_2 progeny). As expected from the 3:1 segregation pattern, we were able to obtain homozygotes for the kanamycin-resistance marker among the R_2 progeny of the 3:1 class plants (see Table 1 and protein analysis, below). Pollen from either R_0 plants or from R_1 and R_2 progeny plants was used for the subsequent analyses.

RNA and protein analyses

In order to test whether the aberrant genetic ratios could be correlated with downregulation of the *LAT52* gene, we analyzed mRNA and protein levels in pollen from wild-type and antisense transformants. Total RNA extracted from mature pollen from seven 3:1 class plants and from 14 1:1 class plants was analyzed by Northern blot hybridization. The results are included in Table 1 and one RNA blot is shown in Figure 4. Most of the transformed plants with normal segregation (3:1 class) had no reduction in *LAT52* mRNA levels, when considering the VF36 control '*LAT52* mRNA/28S rRNA' ratio as 1.00. We do not have an explanation for the reduced levels of *LAT52* mRNA in plants 2, 9 and 11 (Table 1), but both the genetic data and the phenotypical analyses (see below) support that pollen from these 3:1 class plants is functional. On the other hand, all tested plants from the abnormal segregation class (1:1 class) showed a specific reduction in the endogenous message.

Table 1. Summary of results of genetic segregation tests, RNA and protein levels and pollen phenotypes of LAT52 antisense plants

Plant no.	Marker transmission scoring[a]			Copy no.[c]	*LAT52* mRNA[d]	LAT52 protein[e]	Size diff.[f]
	Self-cross						
	R_1	R_2	Backcross[b]				
1	101:51	205:64		1			No
2	27:6	80:29		ND	0.60[2]	1.4[4]	No
		212:0		ND		0.80[8]	
3	135:54			1			No
4	147:57	108:0	36:43	1	0.94[3]		No
5	259:84	241:83	3:5	ND		0.85[8]	No
		274:90		1			
6	311:119	241:0	36:33	ND			No
		89:23		ND			
7	153:59	104:40	13:16	ND			No
		157:55		ND			
8	160:59	67:25		ND			No
9	272:75	176:43		2	0.8[1]	1.02[7]	No
		113:0		ND	0.7[3]	1.20[4]	
						0.92[8]	
10	2:0	121:40		ND			No
11	249:88	163:56		1	0.68[1]	0.70[5]	No
		435:0		ND			
12	125:46	230:74		ND			
13	274:107	216:52		ND			No
14	301:113	189:65		1	0.76[3]	1.07[5]	No
						0.92[6]	
15	117:72	167:59		ND			No
		145:41		ND			
16	220:66	259:46	52:51	ND	0.76[1]		No
		33:0		ND			
17	81:15	171:75		2			No
		220:132		2			
18	272:134		31:28	ND	1.1[3]	0.65[6]	No
					0.8[1]		
19	202:101	87:33	34:31	ND			No
		126:26		ND			
20	538:457		0:7	1		0.55[8]	Yes
21	75:73	193:196	0:18	1			No
22	53:43	152:135		ND			Yes
23	128:107	47:50		ND	0.5[1]	0.40[6]	Yes
					0.5[3]	0.30[4]	
24	264:301			ND			No
25	32:26			ND			No
26	36:38	143:129		1	0.41[1]	0.36[5]	Yes
					0.53[2]	0.11[6]	
					0.48[3]	0.53[8]	

27	51:43	36:37		ND			
28	128:127	31:29 39:34	0:65	ND 2		0.46[8]	Yes
29	182:151	60:67 126:133	0:6	ND 2		0.30[4]	No
30	76:76	44:49	0:17	ND	0.16[3] 0.45[1]	0.30[6] 0.50[4]	Yes
31	207:208	139:136 135:103 25:54		ND ND 1			Yes
32	8:22	376:347		ND	0.15[2]		Yes
33	28:21	32:24 59:64	0:47	ND 4			Yes
34	212:206	157:132 11:20		2 ND			Yes
35	99:103			ND	0.39[1]	0.30[4]	Yes
36	28:34			ND			Yes
37	245:234	140:138 158:131		ND ND	0.30[3]		Yes
38	99:127	418:381		1	0.47[3] 0.34[1]	0.42[5] 0.49[4] 0.55[8]	Yes
39	170:157		0:50	ND	0.27[3]	0.21[6] 0.20[4]	Yes
40	26:49	122:94		ND	0.35[3]		Yes
41	29:25	54:31		ND	0.52[2]		Yes
42	231:238		0:5	2		0.45[8]	No
43	31:5	151:143 156:141		3 ND		0.35[8]	Yes
44	14:2	232:220		1		0.32[5] 0.20[7]	Yes
45	48:55	282:362		1	0.34[1]		Yes
46	36:103	114:106 18:20		1 1	0.33[3]	0.45[4]	Yes
47	206:108	93:98 103:117		ND ND			Yes
48	14:47	472:421		ND	0.38[2]		Yes
49	74:246	435:469		1	0.40[1]	0.16[4] 0.55[8]	Yes
50	48:40	170:174 180:78	0:3	1 ND			Yes
51	3:2	98:210 11:12		ND 1		0.65[8]	Yes

Plants 1–19 are considered as 3:1 class plants and plants 20–51 as 1:1 class plants.
[a]As a 'Kanamycin-resistant plants' to 'Kanamycin-sensitive plants' ratio.
[b]Backcrosses of R_0 plants as male to untransformed females.
[c]As additional DNA bands not present in non-transformed control.
[d]Considering VF36 control '*LAT52* mRNA/28S rRNA' ratio as 1.00.
Superscript numerals (1–3) indicate separate experiments.
[e]Considering V36 control 'LAT52 protein/ubiquitin protein' ratio as 1.00.
Superscript numerals (4–8) indicate separate experiments.
[f]In complete germination medium (see Experimental procedures).

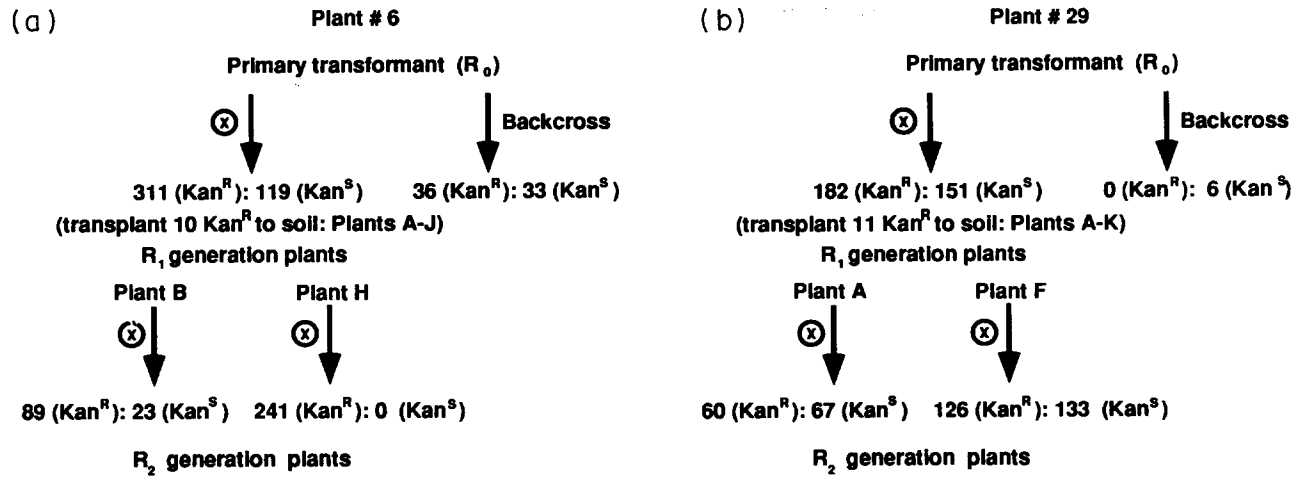

Figure 2. Flow-chart of genetic analyses for LAT52 antisense plants.
(a) Analysis of plant 6, one of the 3:1 class segregation plants. (b) Analysis of plant 29, one of the 1:1 class segregation plants.

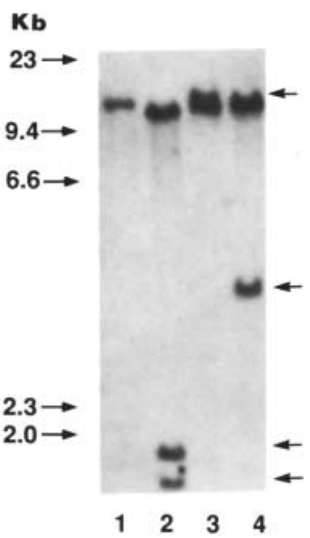

Figure 3. Southern blot of normal and transgenic tomato plants transformed with pLAT52.AS.
Bands corresponding to copies of the antisense construct are indicated with arrows. Lane 1; VF36 (untransformed tomato plant); lane 2, plant 9; lane 3, plant 38; and lane 4, plant 49.

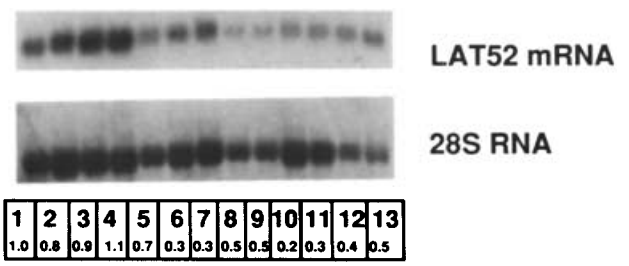

Figure 4. Northern blot analysis of *LAT52* mRNA levels in pollen of LAT52 antisense plants.
The band intensity was scanned with a densitometer and internally corrected by comparison with ribosomal RNA hybridization. Lanes 2–5 are 3:1 class plants and lanes 6–13 are 1:1 class plants.
Lane 1, VF36 (untransformed tomato plant); lane 2, plant 14; lane 3, plant 4; lane 4, plant 18; lane 5, plant 9; lane 6, plant 37; lane 7, plant 46; lane 8, plant 26; lane 9, plant 23; lane 10, plant 30; lane 11, plant 39; lane 12, plant 40; lane 13, plant 38.

In order to characterize the LAT52 protein it was necessary to obtain antisera that would detect the LAT52 protein. Antisera were raised against an *Escherichia coli* overproduced trpE-fusion protein, as described in Experimental procedures. The polyclonal antibody (Ab 52.1) gave a single band in pollen extracts on SDS–PAGE gels, and no signal was detected in other tomato sporophytic tissues, including petal, sepal, leaf, pistil and immature seed (data not shown). The antibody did not recognize the native configuration of the protein on native PAGE blots (data not shown).

Western blot data for six 3:1 class plants and 15 1:1 class plants are included in Table 1 (an example is shown in Figure 5). Western blots prepared with pollen extracts from the 3:1 class of plants showed levels of LAT52 protein equivalent to untransformed control pollen extracts (except for plants 11 and 18), while lower amounts of LAT52 protein were detected in all the 1:1 segregation class plants that were examined, when considering the VF36 control 'LAT52 protein/Ubiquitin protein' ratio as 1.00 (Table 1). Lane 3 of Figure 5 shows that the R_2 homozygote progeny of plant 2 showed normal levels of LAT52 protein, indicating that even though all these pollen grains carry the antisense construct, there was no

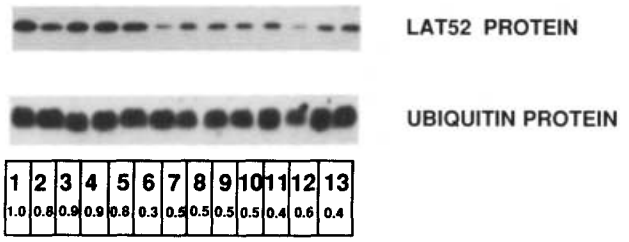

Figure 5. Western blot analysis of LAT52 protein levels in pollen of LAT52 antisense plants.
The blot was sequentially probed with the LAT52 antibody and the ubiquitin antibody. Lanes 2–5 are from 3:1 class plants and lanes 6–13 are from 1:1 class plants.
Lane 1, VF36 (untransformed tomato plant); lane 2, plant 2; lane 3, plant 2 (R_2 homozygous progeny); lane 4, plant 9; lane 5, plant 5; lane 6, plant 43; lane 7, plant 38; lane 8, plant 49; lane 9, plant 20; lane 10, plant 26; lane 11, plant 28; lane 12, plant 51; lane 13, plant 42.

inhibition in the levels of the LAT52 protein. Furthermore, all of the homozygous plants derived from the 3:1 class plants had functional pollen that were able to fertilize and generate seeds in self-crosses (data not shown). This implies that the presence of the antisense gene is necessary but not sufficient to produce a decrease in the levels of the sense message.

Taking all of these results together, there is an obvious correlation between the downregulation of *LAT52* message and protein levels and the genetic data.

Pollen phenotypes

We examined the pollen phenotypes of the transformed plants in both the 1:1 and the 3:1 classes. Dry pollen showed no obvious differences from wild-type, at the light microscope level (data not shown). Upon hydration in pollen germination medium (Jahnen *et al.*, 1989) we noticed that most of the 1:1 class plants (26 of 31 tested) showed two phenotypic classes of pollen, large and round, and small and collapsed. No differences from wild-type were noticed in pollen from the 3:1 class plants (compare Figure 6a and 6b with c; see Table 1). The pollen germination medium is composed of sucrose, MES buffer, salts and PEG 3550. Interestingly, this size difference was not seen when the abnormal shape pollen grains were transferred to a pollen germination medium with no PEG 3550, or to a MES buffer (example shown in Figure 6h and j). In these solutions, the abnormal pollen grains still looked smaller than the normal ones, but rounded up, suggesting that the difference involved some step in the hydration process. The collapsed shape was restored when the smaller rounded ones were transferred back to the original germination medium (Figure 6l). This indicates that the net flux of the water in the abnormal pollen grains is reversible. In contrast, the normal shape pollen grains from the 1:1 class plants did not change shape after initial hydration (Figure 6g, h, j and l; see arrows).

We used cytochemical stains to address the possible disfunction of the LAT52 antisense pollen. The integrity of intine and exine in the pollen grain was determined with the stains tinopal and D_iOC_2 (Regan and Moffatt, 1990). Tinopal binds to the cellulose cell wall of the intine and fluoresces blue. D_iOC_2 stains the exine brillant red. Other than the difference in hydration, there is no apparent difference in intine or exine staining between control or 3:1 class pollen (Figure 6c) and the 1:1 class pollen (Figure 6b).

DAPI binds to double-stranded DNA and fluoresces blue under ultraviolet light (Regan and Moffatt, 1990). There were no differences in nuclear composition or DNA staining between the abnormal shape and normal pollen grains. Figure 6(d) shows one normal (see arrow) and three abnormal shape pollen grains from a 1:1 segregation class plant. DAPI staining of these pollen grains is shown in Figure 6(e). The larger and more diffusely staining vegetative nucleus is seen in both size classes, but because of the plane of focus, the elongated generative nucleus is only visible in one of the abnormal shape pollen grains. Toluidine blue (TBO) is a non-specific stain that binds to the majority of the cellular components, thus staining the cytoplasm blue. TBO staining indicated that the abnormal pollen grains (arrows in Figure 6f) possess cytoplasm. The more intense staining in the abnormal shape grains is presumably due to the incomplete hydration of these grains (Figure 6f).

We used two methods of vital staining, Alexander's stain (data not shown) and fluorescein diacetate (FDA) (Figure 6i and k). Alexander's stain (Alexander, 1987) is composed of four dyes that stain nuclear material and cell wall: living pollen stains purple, and dead pollen stains turquoise. In our hands, Alexander's stain was not compatible with the pollen germination medium we use to distinguish the two size classes of pollen, because lipoidal globules form when the stain is mixed with germination medium containing PEG. We therefore could not directly show that the abnormal shape pollen grains stain purple. However, since 50% of the pollen has an abnormal shape, and more than 90% of the pollen stains purple, we infer that the abnormal shape pollen is alive. Fluorescein diacetate staining is the basis for the fluorochromatic reaction, which occurs when an esterase cleaves the non-fluorescent FDA to release fluorescein (Heslop-Harrison *et al.*, 1984). Because of the reportedly high levels of exine autofluorescence in mature pollen that might interfere with assessment of pollen viability, we also included several controls. Figure 6(m) shows FDA staining of non-transformed control pollen, while Figure

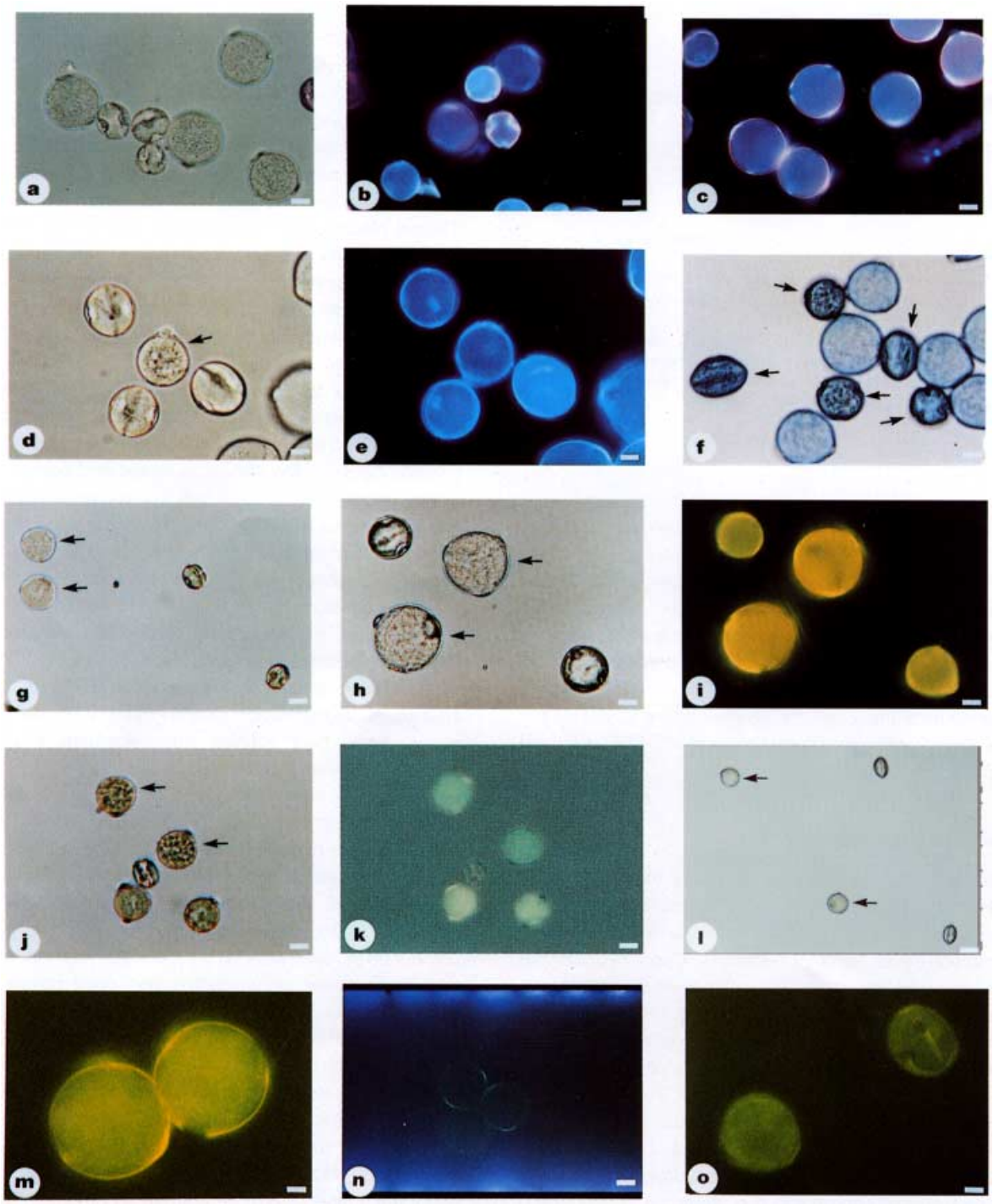

Figure 6. Histochemical stains and hydration characteristics of LAT52 antisense pollen.
(a) Plant 49 pollen (1:1 class) in pollen germination medium. (b) Plant 49 pollen stained for exine and intine. (c) Plant 9 (3:1 class) pollen stained for exine and intine. (d) Plant 20 pollen (1:1 class) in pollen germination medium. (e) DNA staining of 'd'. (f) Plant 20 pollen stained with TBO. (g) Plant 49 pollen in pollen germination medium. (h) Pollen from 'd' transferred to pollen germination medium with no PEG. (i) FDA staining of 'h'. (j) Pollen from 'h' transferred to MES buffer. (k) FDA staining of 'j'. (l) Pollen from 'j' transferred back to pollen germination medium. (m) FDA staining of untransformed (VF36) pollen. (n) Exine autofluorescence of untransformed (VF36) pollen. (o) FDA staining of heat-killed untransformed (VF36) pollen.
Bars: 12 μm (a, b, c, d, e and f); 17 μm (g, j and k); 11 μm (h, i and o); 32 μm (l); 6 μm (m); 15 μm (n). Arrows in (d), (g), (h), (j) and (l) indicate normal shape pollen grains, and arrows in (f) indicate abnormal shape pollen grains. A dead pollen grain was included in (j) and (k) as a negative control. The photograph in (n) was taken with Ektachrome 160 ASA film.

6(n) shows pollen autofluorescence and Figure 6(o) shows FDA staining of heat-killed pollen.

In vitro pollen germination revealed differences between the 1:1 class and untransformed control plants. Depending on which plant was examined, about 50% of the pollen from the 1:1 class plants either did not germinate at all, or if it did germinate, had corkscrew pollen tubes (data not shown), while no particular difference from control pollen-tube growth was seen when pollen from 3:1 class plants was germinated *in vitro* (data not shown). *In vivo* germination of pollen also revealed differences. In the 1:1 class plants (three examples of 10 different tested plants are shown in Figure 7c, d and e) some of the pollen tubes grew normally down the style, while others twisted and turned, and often appeared to tie themselves in knots (Figure 7f). In contrast, pollen from 3:1 class plants (one example of seven different tested 3:1 class plants is shown in Figure 7b) exhibited no apparent differences from control pollen (Figure 7a) when germinated *in vivo*.

LAT52 protein characterization

We attempted to immunolocalize the LAT52 protein, but were unsuccessful, probably because the LAT52 antibody (Ab 52.1) does not recognize the native configuration of the protein. We therefore synthesized the LAT52 protein in a baculovirus expression system in order to address partially the questions of cellular localization and glycosylation.

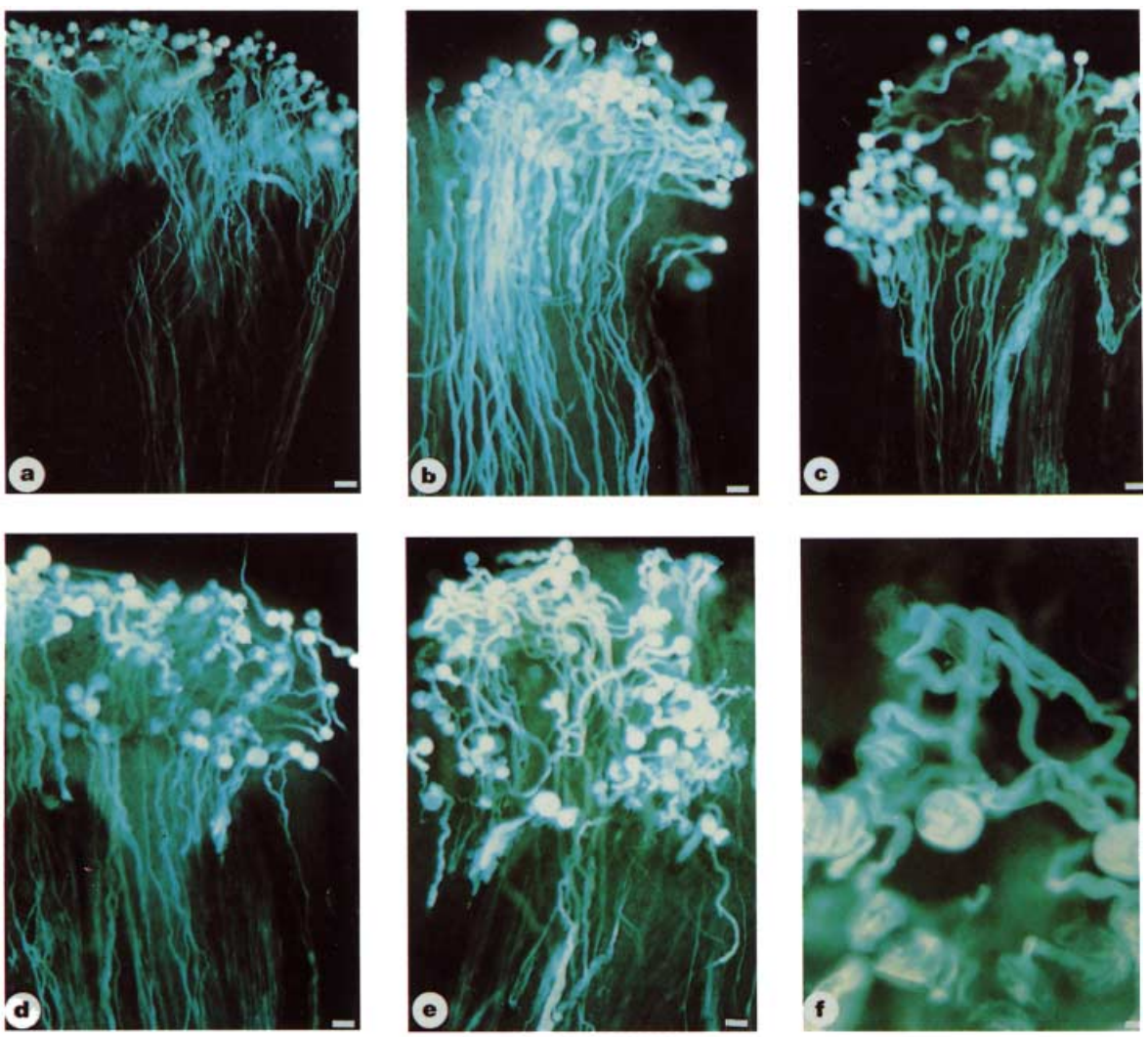

Figure 7. *In vivo* pollen germination assays of LAT52 antisense plants.
VF36 pistils were pollinated and stained 24 h. later with aniline blue. (a) Control untransformed VF36 pollen. (b) Plant 6 pollen (3:1 class). (c) Plant 49 pollen (1:1 class). (d) Plant 26 pollen (1:1 class). (e) Plant 20 pollen (1:1 class). (f) Close-up of pollen tubes from 'e'. Bars: 35 μm (a, b, c, d, and e); 10 μm (f).

Sf9 cells infected with baculovirus containing the *LAT52* gene fused to the polyhedrin promoter produce a protein that is detectable with the LAT52 antibody (Figure 8a). LAT52 protein is detectable in immunoblots of cell extracts at 48 h post-infection (Figure 8a,A), and in immunoblots of culture medium at 72 h post-infection (Figure 8a,B). The LAT52 protein in the tissue culture medium does not come from cell lysis, because on the Coomassie-stained gel other prominent cellular proteins are absent from the medium fraction. No protein is detectable with the antibody in extracts prepared from uninfected Sf9 cells, or from cells infected with a baculovirus lacking the *LAT52* coding sequence (data not shown).

In order to determine whether LAT52 was glycosylated on the potential N-linked glycosylation site, we treated cell extracts and culture medium fractions with endoglycosidase (endo) H, which cleaves high-mannose oligosaccharides from proteins, and endo F, which cleaves both high-mannose and complex oligosaccharides from proteins. The intracellular LAT52 is sensitive to both endo H and endo F (Figure 8b, A), whereas the extracellular protein is resistant to endo H and sensitive to endo F (Figure 8b,B). LAT52 in pollen is also endo H-resistant and endo F-sensitive (data not shown). These results are consistent with the interpretation that LAT52 in insect cells and in pollen traverses the secretory pathway, at least as far as the Golgi, where endo H-sensitive high-mannose oligosaccharides are converted to endo H-resistant complex oligosaccharides.

LAT52 and the pollen proteins from maize, rice and olive share sequence similarity with several Kunitz trypsin inhibitors (Jofuku and Goldberg, 1989; McCormick *et al.*, 1991a). An alignment of the pollen proteins and the soybean Kunitz trypsin inhibitor 1 is shown in Figure 9. Many of the known plant protease inhibitors are rich in cysteine residues and are extremely resistant to denaturation by heat (Richardson, 1977), while most soluble proteins are heat sensitive and precipitate upon boiling of cell extracts. Because of its sequence similarity to proteinase inhibitors, we tested the heat stability of LAT52. Figure 10 shows that the LAT52 protein remains soluble upon heating in the absence of SDS, while most other pollen proteins were denatured and precipitated. Kunitz trypsin inhibitor proteins typically contain four conserved cysteine residues which form two intracellular disulfide bridges (Kortt *et al.*, 1990). Immunoblot analyses of pollen extracts prepared in the presence or absence of DTT indicated the existence of disulfide bridges in the LAT52 protein (data not shown). While these four pollen proteins share five conserved cysteine residues, only two of these are conserved between the pollen proteins and the Kunitz inhibitors, indicating that the positions of disulfide bridges in the pollen proteins are likely to be different.

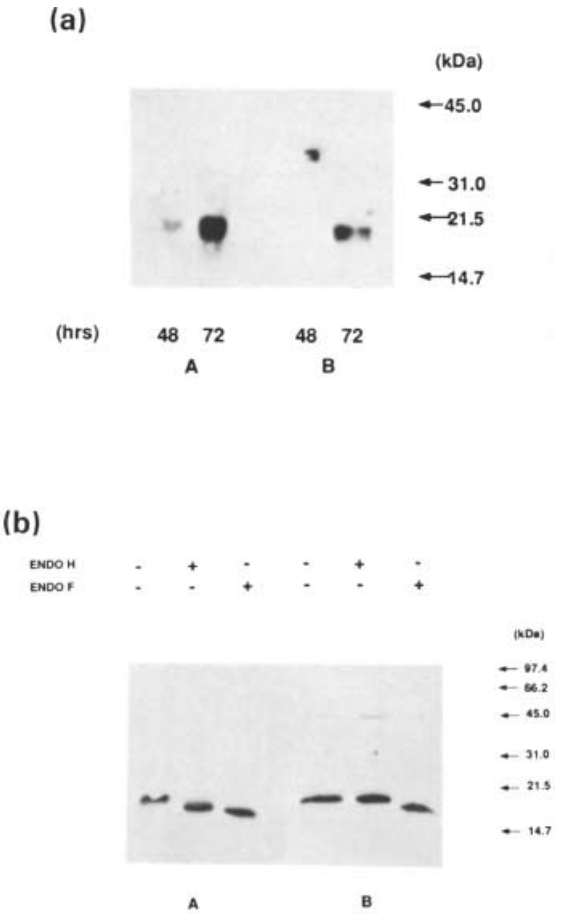

Figure 8. Expression of LAT52 in Sf9 cells infected with 52-BAC.
(a) Western blot of LAT52 protein secreted in insect cells. Total cell extracts (A) and supernatants (B) were separated from cells at the indicated times post infection and analyzed by SDS–PAGE and immunoblotting with Ab52.1. as described in Experimental procedures.
(b) Western blot of endo H-/endo F-sensitivity assay: 72 h post-infection insect cell extracts (A) or cell supernatants (B) were incubated in the presence of endo H, endo F, or neither, as described in Experimental procedures. Samples were analyzed by SDS–PAGE and immunoblotting with Ab52.1.

Discussion

The results presented in this paper demonstrate that pollen with reduced expression of the *LAT52* message and protein develops abnormally and is non-functional. In other antisense experiments (e.g. Riesmeier *et al.*, 1993; Stockhaus *et al.*, 1990; Tieman *et al.*, 1992; Zhang *et al.*, 1992) only a small fraction of the plants show a phenotype that is correlated with expression of the antisense construct. Our conclusions are strongly supported because the majority of the transformants with reduced message and protein show the abnormal pollen phenotype. The LAT52 antisense pollen phenotype differs from those seen with the *pop1* mutation (Preuss *et al.*, 1993), the

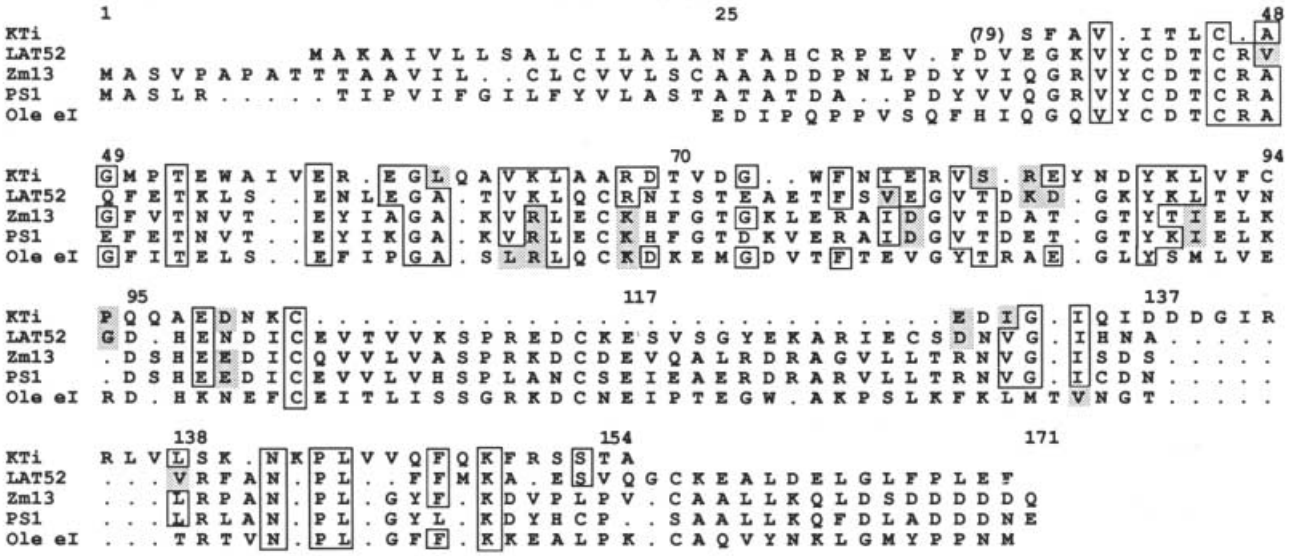

Figure 9. Sequence comparisons of pollen proteins and a proteinase inhibitor protein.
Alignment of the tomato LAT52, rice PS1, corn Zm13 and Ole e I proteins with the soybean Kunitz trypsin inhibitor 1 (KTi1). Amino acid numbering is relative to the Zm13 protein. Identical amino acids between the pollen proteins and KTi1 are outlined with open blocks, whereas conservative amino acid exchanges [(K,R), (E,D), (Q,N), (S,T), (P,G), (M,C), (F,Y,W,H) and (L,I,V,A)] are shaded. Gaps introduced to optimize the alignment are marked by (.).

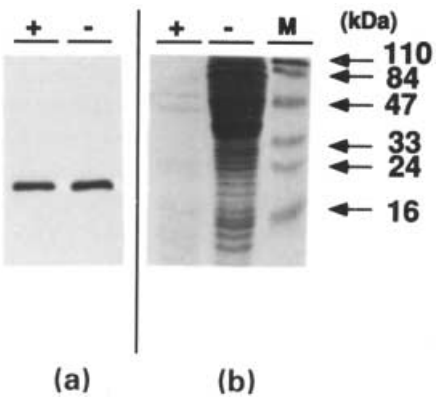

Figure 10. Heat stability of the LAT52 protein.
(a) Immunoblot analysis of tomato (cv. VF36) pollen using Ab52.1.
(b) Coomassie blue stained SDS–PAGE gel (identical to that shown in a). Pollen extracts (70 μg protein) were loaded in the '–' lanes, while in the '+' lanes an equivalent amount was heated for 2 min at 90°C in the absence of SDS before loading. Lane 'M' represents molecular weight standards.

aprt– mutation (Regan and Moffatt, 1990), and the white pollen mutations (Mo *et al.*, 1992), all of which are sporophytically acting and affect all the pollen of an affected plant, as well as other parts of the plant. Because *LAT52* has a gametophytic expression only the pollen grains that carry the antisense construct can express the abnormal phenotype. The LAT52 abnormal shape antisense pollen, unlike *pop1* pollen, could not be rescued *in vitro* under high-humidity conditions, or with the help of mentor pollen *in vivo*. In fact, if mentor pollen had been effective in rescuing the LAT52 abnormal shape antisense pollen grains, we would never have noticed the altered segregation pattern in self-progeny of the 1:1 class plants, since in each such self-pollination the 50% normal pollen could potentially serve as helper pollen.

Antisense approach

Most antisense experiments have studied proteins of known function (e.g. Hall *et al.*, 1993; Oeller *et al.*, 1991; Rodermel *et al.*, 1988) and have demonstrated a correlation between downregulation of the sense message and a reduction of the enzymatic activity. However, there are some reports where antisense constructs were used to assign a function to unknown proteins. Such experiments showed, for example, that the pTOM13 protein is involved in ethylene synthesis (Hamilton *et al.*, 1990), that the pTOM5 protein is part of the carotenoid synthesis pathway (Bird *et al.*, 1991) and that a 10 kDa protein might play a role in the water-splitting apparatus of photosystem II (Stockhaus *et al.*, 1990).

We used the *LAT52* promoter to drive expression of the *LAT52* antisense gene. This promoter was chosen for two reasons: (i) the commonly used CaMV 35S promoter is poorly expressed in pollen (Twell *et al.*, 1989a) and (ii) using the *LAT52* promoter would insure that the antisense promoter would be as strong as the endogenous

gene promoter. This consideration is relevant because the proposed model for inhibition of gene expression by antisense RNA is hybrid formation between the sense and antisense RNA (Temple *et al.*, 1993). If there is insufficient antisense RNA present in the cell, the remaining sense RNA might generate enough protein to produce a normal phenotype (Palomares *et al.*, 1993; Riesmeier *et al.*, 1993). Our results and those of some others (van der Krol *et al.*, 1990b; Robert *et al.*, 1989) demonstrate that the use of the homologous promoter to drive expression of the antisense gene is sufficient to inhibit the sense message.

Of necessity, our antisense analyses were performed on plants heterozygous for the *LAT52* antisense gene, where *LAT52* levels are expected to be normal in 50% of the pollen. Taking this into account, we can conclude that the plants from the 1:1 segregation class must show a nearly complete reduction of the sense message in the pollen that carries the *LAT52* antisense construct, because the *LAT52* mRNA that these plants have (50% or less, compared with control untransformed plants) presumably comes from the half of the pollen that does not carry the antisense gene. Thus, the only pollen that is functional does not carry the antisense construct, and it was impossible to obtain homozygotes for the linked kanamycin-resistance gene in the progeny of these 1:1 class plants. On the other hand the plants in the 3:1 segregation class showed normal levels of *LAT52* message and a normal pollen phenotype, and we obtained homozygotes among their progeny. We presume that these 3:1 class plants must not express enough of the antisense message to have a significant effect on the sense expression of *LAT52*, presumably because of a position effect on expression of the transgene (van der Krol *et al.*, 1990a; Cannon *et al.*, 1990).

LAT52 protein characterization

Our LAT52 antibodies do not recognize native LAT52, which precludes their use for immunolocalization studies. We have expressed LAT52 in insect cells under the control of the baculovirus polyhedrin promoter in order to determine if the protein is a secretory protein. Several plant proteins known to normally enter the secretory pathway, for example, phaseolin and papain (Bustos *et al.*, 1988; Vernet *et al.*, 1990), have been demonstrated to be translocated across the ER membrane and to travel through the Golgi in insect cells, as assayed by acquisition of high mannose and complex glycosylations. LAT52 expressed in insect cells enters the secretory pathway and acquires high mannose (endo H-sensitive) and complex (endo H-resistant) glycosylations (Figure 8b). LAT52 in pollen also has complex glycosylations. Late in infection more LAT52 accumulates intracellularly than is secreted (Figure 8a), which might argue for an intracellular location in insect cells. However, Jarvis and Summers (1989) found that secretion of human tissue plasminogen activator becomes less efficient as infection progresses, which suggests that secretory function decreases late in viral infection. So while we can conclude that the LAT52 protein traverses the secretory pathway, from these results alone we cannot definitely say whether LAT52 is secreted in pollen, because some vacuolar or lysosomal proteins are secreted when they are overexpressed in insect cells (Bustos *et al.*, 1988, Itoh *et al.*, 1990). The pollen allergen from olive tree is similar to LAT52 (Figure 9), and is glycosylated (Batanero *et al.*, 1994). Because allergens are generally considered to be pollen surface localized, we think it is likely that LAT52 is also secreted.

LAT52 shows sequence similarity to proteinase inhibitors

The fact that LAT52, Zm13, Ole e I and PS1 have similar protein sequences and that the transcripts have identical tissue-specific expression patterns support the idea that these genes encode functionally homologous proteins. Because of the large evolutionary distance between these species, the amino acids that are conserved are probably important for the structure or function of these proteins. All four pollen proteins show sequence similarity to several Kunitz trypsin inhibitors. These similarities are dispersed throughout the central and carboxy-terminal part of the inhibitor proteins (Figure 9), but do not include the active site domain, which is found in active proteinase inhibitors of the Kunitz-type at amino acids 63–64. This might imply that the pollen proteins are not active proteinase inhibitors. However, in at least one case, an active cysteine protease inhibitor in pig leucocytes lacks some of the conserved amino acids previously thought to be required in its active site (Ritonja *et al.*, 1989).

There are other examples of proteins that have sequence similarity to Kunitz-type proteinase inhibitors, but that lack activity; for example, the soybean proteins KTi1 and Kti2 (Jofuku and Goldberg, 1989). Winged bean albumin (WBA) is a seed protein that shows sequence similarity to both the Kunitz-type family and to the 2S class of storage proteins (Kortt *et al.*, 1989), but has no detectable inhibitor activity. In mammalian sperm numerous protease inhibitors have been found (e.g. Falase *et al.*, 1991), and are assumed to protect the epididymal epithelium against the proteolytic action of enzymes released from dead sperm. Similar to the seed storage protein case, there are sperm-specific small heat-stable or cysteine-rich proteins that show sequence similarity to proteinase inhibitors, but which do not conserve the active site domain thought to be required for activity (e.g. Cornwall *et al.*, 1992; Kirchoff *et al.*, 1991). It

has been suggested that Kunitz-type family members which lack activity as proteinase inhibitors may play a storage protein role (Jofuku and Goldberg, 1989). We plan to test whether LAT52 is an active proteinase inhibitor by purifying it from insect cells expressing the 52-BAC construct or from mature pollen.

Protease inhibitors present in *Nicotiana* stigmas were suggested (Atkinson *et al.*, 1993) to play a defensive role in the stigma against pathogens. If we consider LAT52 as a protease inhibitor, its function could be to regulate a protease activity during pollen development, thereby protecting from degradation other proteins that are critical for pollen hydration or pollen germination. Although there is very little information about the need for protease inhibitors in pollen, proteolytic activity was associated with the intine of the pollen of some species, near the sites of emergence of the pollen tube (Knox and Heslop-Harrison, 1970; Knox *et al.*, 1975). It is also possible that pollen proteinase inhibitors might protect pollen tubes from style-secreted proteases.

Pollen hydration and pollen-tube growth

It is thought (Heslop-Harrison, 1987) that pollen hydration occurs because of differences in water potential between the stigma (or the medium, in an *in vitro* assay) and the pollen. Bashe and Mascarenhas (1984) reported that protein synthesis in Tradescantia pollen began within 2 min of hydration, and they speculated that the decrease in K^+ concentration in the pollen cytoplasm (due to the entrance of water) is responsible for the activation of protein synthesis. When hydration is completed, the vegetative cell becomes an osmotic system, bounded by a semipermeable membrane contained within an elastic pollen cell wall. The regions of the pollen cell wall responsible for water movement are the aperture sites; in these regions (the potential points of exit for the pollen tube) the exine is reduced or absent and the intine expands in thickness (Polowick and Sawhney, 1993).

We suggest that the shape of the abnormal pollen is indicative of hydration problems, but we can not conclude that the disfunction generated by the introduction of the *LAT52* antisense gene directly causes a defect in hydration. The more complete hydration that we saw when abnormal pollen grains were incubated in a germination medium with no PEG or in a MES buffer (media with higher water potential than the germination medium with PEG) refutes the idea that the disfunction resides in the entrance of the water into the grain. As we showed, the abnormal shape was restored when these partially hydrated antisense pollen grains were re-incubated in complete germination medium. This suggests that the problem might reside in some process related to water retention. We detected no gross abnormalities in the intine layer of the abnormal pollen grains by tinopal staining, but cannot rule out the possibility of intine involvement, because LAT52 may be secreted and might be intine localized. It will be necessary to obtain antibodies that will recognize the native configuration of the LAT52 protein, in order to test whether LAT52 is intine localized and whether it is concentrated in the aperture regions.

The pollen tube is an extension of the intine. The pollen tube extends by tip growth (Steer and Steer, 1989) and its wall is largely composed of callose (Schlüpmann *et al.*, 1993). The abnormal antisense LAT52 pollen grains can germinate poorly if at all *in vitro*, and somewhat better *in vivo*, but tube growth is abnormal and arrests in the style. Perhaps the poorer germination of the antisense pollen can also be explained in terms of pollen hydrostatic pressure. If pollen-tube emergence and subsequent tip growth requires a considerable hydrostatic pressure that is only generated by complete hydration, then the antisense pollen grains cannot attain the pressure threshold required to generate normal pollen tubes that are able to grow correctly toward the ovary (Figure 7c, d, e and f). Support for this hypothesis comes from analyses of heterostyly incompatibility in *Primula* (Shivanna *et al.*, 1983), where incompletely hydrated pollen grains developed abnormal pollen tubes.

The abnormalities in the germination of the 1:1 class pollen *in vivo* are also reminiscent of pollen-tube growth in incompatible crosses. For example, interspecific crosses in *Rhododendron* show various types of arrested pollen tubes (Williams *et al.*, 1982), which were suggested to be due to either an incorrect synthesis or deposition of polysaccharide, to an osmotic mismatch between the pollen and the pistil, and/or to a change in the polarity of the tip growth. In our abnormal pollen tubes we found some of the phenotypes seen in the *Rhododendron* crosses (Figure 7c, d and e), such as bursting tubes, coiling or spiralling tubes. Shivanna *et al.* (1981) also demonstrated that coiled tubes are characteristic of incompatible reactions. Lastly, Rao and Kristen (1990) found similar screw-like pollen tubes when tobacco pollen was grown in the presence of 25 mg l^{-1} Triton X-100, a non-ionic detergent.

Is there a connection between the sequence similarity to proteinase inhibitors and the hydration defects seen in the LAT52 antisense pollen? One is suggested by the work of Araki *et al.* (1989), who reported the characterization of a family of porcine peptides that negatively regulated a Na^+, K^+-ATPase; their protein structure showed high similarity to some proteinase inhibitors, but did not show any protease inhibitory activity. An H^+-ATPase (Obermeyer *et al.*, 1992) and K^+-channels (Obermeyer and Kolb, 1993) have been found in pollen grains, and they seem to be involved in the regulation of a pollen transcellular current (Weisenseel and Jaffe, 1976).

The outward pollen current is carried by protons while K^+ ions, and to a lesser extent Ca^{2+} ions, are responsible for the inward current. The pollen water potential is controlled in part by the solute balance between the pollen cytoplasm and the external medium. If any of the proteins responsible for this balance (e.g. ATPases, ion channels, or regulators of them) do not act correctly or are absent, a water potential imbalance could generate disfunctions in pollen hydration.

Taking all of our speculations about the function of LAT52 into account (proteinase inhibition, ion channel regulation), it seems most plausible that LAT52 acts in an indirect way in pollen hydration or pollen germination. We plan to test whether LAT52 functions in this manner in the near future.

Experimental procedures

Plant material

Untransformed and transformed tomato (*Lycopersicon esculentum* cv. VF36) plants were grown under standard greenhouse conditions. Self-pollinations occur in the VF36 cultivar without human intervention (Rick and Dempsey, 1969), because the stigma is well inserted within the anther cone. For controlled pollinations, flowers on untransformed VF36 plants were hand emasculated before anther dehiscence and pollinated by applying pollen with a shield-shaped needle to the surface of the stigma. Mature pollen was collected by vibrating the anthers and was used directly for germination tests and cytological analyses, or stored at –80°C for RNA or protein extract preparations.

Construction of LAT52 *antisense gene, plant transformation and genetic analysis*

Promoter 'strength' is an important consideration when designing antisense experiments. For example, the commonly used CaMV 35S promoter is poorly expressed in pollen (Twell *et al.*, 1989a). Of the three pollen genes that we analyzed (Twell *et al.*, 1991), the *LAT52* promoter is the most strongly expressed (McCormick *et al.*, 1991b). Reasoning that the antisense promoter should be at least as strong as the endogenous gene promoter and that it should overlap the time of expression, we used the *LAT52* promoter to drive expression of the *LAT52* antisense transcript.

This was constructed by placing the entire *LAT52* cDNA in antisense orientation and the 3′ end of the *NOS* gene (*Hin*dIII–*Bam*HI restriction sites) behind the 3 kb *LAT52* promoter (Twell *et al.*, 1991); this construct was transferred to pBin19, and finally to *Agrobacterium* strain LBA4404. Tomato (cv. VF36) cotyledons were transformed as described in McCormick (1991a). Seeds were plated on 1/2× MSO medium (McCormick, 1991a) containing 100 μg ml^{-1} kanamycin, and scored for resistance (branched roots) versus sensitivity (stunted roots) 10–14 days later. Genetic ratios were statistically analyzed with the chi-square test. R_1 and R_2 progeny were transplanted to soil from kanamycin medium. For each primary transformant, due to constraints on greenhouse space, usually only one to three plants of the R_1 or R_2 generation were grown to mature plant stage for fruit set and seed production.

Overexpression of LAT52 in Escherichia coli *and antibody production*

A trpE fusion protein expression system (Dieckmann and Tzagoloff, 1985) was used to produce antigens for antibody production. The entire translated portion of the cDNA (161 amino acids) was used. For the *trpE–LAT52* construct, the 515 bp *Aha*III–*Hin*dIII fragment from the cDNA p*LAT52* (Twell *et al.*, 1989a) was ligated into the *Sma*I–*Hin*dIII sites of pATH2. The construct was transformed in the bacterial host strain XL1 Blue (Stratagene, La Jolla). The induction and partial purification of the fusion protein was performed according to Spindler *et al.* (1984). The protein was further purified by SDS–PAGE. The molecular weight of the fusion protein was 54 kDa, as predicted. The protein was eluted from excised gel fragments of SDS–PAGE gels and used to immunize Swiss Webster mice (Harlow and Lane, 1988). Approximately 20 mg protein in incomplete Freund's adjuvant was used per immunization for four boosts. The immune response was monitored by ELISA on tail bleeds, 10 days after immunization. Ascites fluids were induced by the sarcoma line T180 (ATCC #TIB 66), and were collected approximately 7, 10 and 13 days after immunization. Ascites fluids were used directly for immunodetection (1:1000 dilution).

Construction of LAT52*-containing recombinant baculovirus*

A 484 bp fragment of the *LAT52* cDNA, extending from –4 to +480, was ligated into the blunted *Bam*HI site of pVL941 (O'Reilly *et al.*, 1992). This fragment encodes all of *LAT52* except the stop codon, which is provided by the pVL941 vector. An additional 20 amino acids are encoded between the end of *LAT52* and the stop codon. This vector is designated pVL52. *Spodoptera frugiperda* cells (Sf9 cells) were grown in Grace's Insect Cell culture medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS), and were co-infected with pVL52 and linearized Baculogold virus DNA (Pharmingen) according to the manufacturer's instructions. Recombinant virus (52-BAC) was isolated after two plaque purifications by standard methods (O'Reilly *et al.*, 1992). The 'no insert' control was made by co-infecting the pVL941 vector with linearized Baculogold viral DNA.

DNA isolation and hybridizations

Genomic DNA was extracted from frozen leaves as described (Bernatzky and Tanksley, 1986). Approximately 3 μg of genomic DNA were digested with *Hin*dIII, separated on a 0.8% agarose gel and blotted to Nytran membranes using standard conditions (Sambrook *et al.*, 1989). The membranes were hybridized at 65°C with random primer-labeled *LAT52* cDNA sequence (Twell *et al.*, 1989b), in 5× SSPE (1× is 150 mM NaCl, 10 mM Na phosphate, pH 7, 1 mM EDTA), 2× Denhardts solution (0.04% Ficoll, 0.04% poly-vinyl pyrrolidine, 0.04% bovine serum albumin), 0.1% SDS and 100 μg ml^{-1} denatured salmon sperm DNA, and washed at 65°C in 0.3× SSPE, 0.1% (w/v) SDS.

RNA isolation and hybridizations

Total RNA from mature pollen was extracted as described (Logemann *et al.*, 1987) and stored as in Chomczynski (1992). RNA was electrophoresed and blotted to Nytran membranes

using standard methods (Sambrook *et al.*, 1989). *LAT52* sense RNA was detected using strand-specific probes of the 0.5 kb fragment of the *LAT52* cDNA clone cloned into pGEM3Z+ (Promega) and labeled with ^{32}P using a Promega *in vitro* transcription system with SP6 polymerase. Similarly a probe that detected 28S ribosomal RNA was prepared using this vector transcribed in the T7 direction. Membranes were hybridized at 65°C using the DNA hybridization conditions, but washed in 1× SSPE, 0.1% SDS at 65°C.

Protein extraction

Mature pollen from transformed plants was disrupted in a glass–glass homogenizer on ice using 1 volume of protein extraction buffer (25 mM phosphate buffer, pH 7.1; 2 mM $Na_2S_2O_5$; 2.4 mM PMSF). The homogenate was centrifuged at 12 000 ***g*** for 10 min at 4°C to remove cellular debris and the supernatant was used immediately for the subsequent experiments. Protein concentration was determined by BCA protein reagent (Pierce) using bovine serum albumin as a standard.

Immunochemistry

Proteins were separated by SDS–PAGE and blotted to nitrocellulose membranes (BA 85, Schleicher & Schuell) by electrotransfer. The membranes were blocked with a suspension of 5% non-fat milk (Harlow and Lane, 1988), incubated with 1:1000 dilutions of Ab52.1 or ubiquitin (Sigma) antisera, and detected using ECL developer following the manufacturer's instructions (Amersham). Molecular weight protein markers (Bio-Rad) were applied on all gels. Densitometry of Northern and Western blots was done using a video camera (Javelin/ultrachip HI-RES CCD, zoom 1.25–70 mm) linked to an Image 1.47 MacIntosh computer program.

Synthesis of LAT52 by Sf9 cells

Sf9 cells were grown in Grace's medium + 10% FCS. Sf9 cells (6×10^5) were infected with 52-BAC at a multiplicity of infection (MOI) of 200 for each time point. At 48 and 72 h post-infection the supernatant was centrifuged to remove any cells and acetone-precipitated. The supernatant precipitate was resuspended or the cells were lysed in 100 µl of SDS–PAGE sample buffer. Equal amounts of cell and medium fractions were separated on a 12.5% polyacrylamide gel, immunoblotted and reacted with Ab52.1.

Endo H-Endo F-sensitivity assays

Sf9 cells, grown in Sf-900II (serum-free medium, Gibco-BRL), were infected as indicated above. At 72 h post-infection the cells were lysed in 1× elution buffer (50 mM Tris, pH 6.8, 0.5% SDS, 100 mM β-mercaptoethanol), and the medium fraction was adjusted to 1× elution buffer. The cell and the medium fractions were boiled for 5 min and an aliquot of each was incubated with either endoglycosidase H or endoglycosidase F (Boehringer Mannheim) according to the manufacturer's instructions.

In vitro *pollen germination and staining*

Pollen germination medium was as described (Jahnen *et al.*, 1989), except that the PEG used (Sigma) has an average molecular weight of 3550 instead of 4000. For the reversible-shape experiment (Figure 6g–l), two abnormal and two normal shape pollen grains (from 1:1 class plant 49) in complete germination medium were transferred using a sequencing pipette tip and examined with a dissecting microscope. After photography, those same pollen grains were transferred sequentially to a germination medium with no PEG, to a MES buffer and finally to complete germination medium again. The germination medium and the MES buffer also contain 0.1% fluorescein diacetate (FDA) in order to check pollen viability. After each transfer the pollen grains were incubated for 10 min before photography. Heat-killed pollen grains were obtained by boiling pollen resuspended in complete germination medium for 10 min.

DAPI (4',6-diamino-2-phenylindole, Sigma) was prepared at 1 mg ml^{-1} solution in 7% sucrose and Toluidine blue (TBO, Kodak) at 0.5% in 1% Boric Acid. $DiOC_2$ (3,3'-diethyloxadicarbocyanine iodide, Sigma) was prepared at 1.0 mg ml^{-1} in distilled water; Tinopal (Sigma) at 1.0 mg ml^{-1} in 500 mM NaCl. The stains were added to pollen that was previously suspended in germination medium, to a final concentration of 2 µg ml^{-1} DAPI, 0.05% TBO, 0.5 µg ml^{-1} $DiOC_2$ or 10 µg ml^{-1} Tinopal. The pollen suspension was allowed to stand at room temperature for 30 min before observation.

Aniline Blue staining of pollinated pistils

Staining was essentially as described in Kho and Baer.(1968). Pistils were collected 24 h postpollination and cut longitudinally in two equivalent pieces. They were fixed in 3:1 ethanol:acetic acid for 1 h at room temperature and softened overnight in 8 M NaOH. The pistils were washed several times with distilled water and incubated with aniline blue (Fisher)(0.1%, dissolved in 0.1 M K_3PO_4), for 3 h in complete darkness. The stained pistils were placed in a drop of glycerol on a microscope slide, carefully squashed under a cover slip and observed with a fluorescence microscope.

UV fluorescence microscope

Mature pollen and pollinated pistils were examined using a Zeiss Photomicroscope set up with a regular halogen lamp or a high-pressure Mercury vapor lamp using a density filter slide A HD. Kodak Ektachrome (400 ASA) film was used for all photography unless otherwise noted.

Acknowledgments

We thank Judy Yamaguchi for making the trpE and antisense constructs, Rachel Holmes and Cherine Fu for DNA analyses, scoring genetic ratios and collecting pollen, David Hantz for excellent greenhouse assistance, Ron Wells for computer advice, Sakis Theologis for helpful suggestions and the members of our laboratory, especially Yoram Eyal and Susan Gorman, for helpful discussions. We also thank Alice Cheung for sharing results prior to publication.

This work is supported by USDA CRIS No. 5335-21000-008-00D, and by the NSF Center for Plant Developmental Biology (UC-Berkeley, DIR 8719933).

References

Alexander, M.P. (1987) A method for staining pollen tubes in pistil. *Stain Technol.* **62**, 107–112.

Araki, K., Kuroki, J., Ito, O., Kuwada, M. and Tachibana, S. (1989) Novel peptide inhibitor (SPAI) of Na^+, K^+-ATPase from porcine intestine. *Biochem. Biophys. Res. Commun.* **164**, 496–502.

Atkinson, A.H., Heath, R.L, Simpson, R.J., Clarke, A.E. and Anderson, M.A. (1993) Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. *Plant Cell*, **5**, 203–213.

Bashe, D. and Mascarenhas, J.P. (1984) Changes in potassium ion concentrations during pollen dehydration and germination in relation to protein synthesis. *Plant Sci. Lett.* **35**, 55–60.

Batanero, E., Villalba, M. and Rodriguez, R. (1994) Glycosylation site of the major allergen from olive tree pollen. Allergenic implications of the carbohydrate moiety. *Mol. Immunol.* **31**, 31–37.

Bernatzky, R. and Tanksley, S.D. (1986) Toward a saturated linkage map in tomato based on isozymes and random cDNA sequences. *Genetics*, **112**, 887–898.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 8711–8721.

Bird, C.R, Ray, J.A., Fletcher, J.D. *et al.* (1991) Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. *Biotechnology*, **9**, 635–639.

Brown, S. and Crouch, M. (1990) Characterization of a gene family abundantly expressed in *Oenothera organenesis* pollen that shows sequence similarity to polygalacturonase. *Plant Cell*, **2**, 263–274.

Bustos, M.M., Luckow, V.A., Griffing, L.R., Summers, M.D. and Hall, T.C. (1988) Expression, glycosylation and secretion of phaseolin in a baculovirus system. *Plant Mol. Biol.* **10**, 475–488.

Cannon, M., Platz, J., O'Leary, M., Sookdeo, C. and Cannon, F. (1990) Organ-specific modulation of gene expression in transgenic plants using antisense RNA. *Plant Mol. Biol.* **15**, 39–47.

Chomczynski, P. (1992) Solubilization in formamide protects RNA from degradation. *Nucl. Acids Res.* **20**, 3791–3792.

Cornwall, G.A., Orgebin-Crist, M.-C. and Hann, S.R. (1992) The CRES gene: A unique testis-regulated gene related to the cystatin family is highly restricted in its expression to the proximal region of the mouse epididymis. *Mol. Endocrinol.* **6**, 1653–1664.

Dieckmann, C.L., and Tzagoloff, A. (1985) Assembly of the mitochondrial membrane system-CBP6, a yeast nuclear gene necessary for synthesis of cytochrome-b. *J. Biol. Chem.* **260**, 1513–1520.

Dubald, M., Barakate, A., Mandaron, P. and Mache, R. (1993) The ubiquitous presence of exopolygalacturonase in maize suggests a fundamental cellular function for this enzyme. *Plant J.* **4**, 781–791.

Falase, E.A.O., Storey, B.T. and Teuscher, C. (1991) Purification and preliminary characterization of guinea pig testicular proteinase inhibitors. *Mol. Reprod. Devel.* **29**, 29–39.

Hall, L.N., Tucker, G.A., Smith, C.J.S. *et al.* (1993) Antisense inhibition of pectin esterase gene expression in transgenic tomatoes. *Plant J.* **3**, 121–129.

Hamilton, A.J., Lycett, G.W. and Grierson, D. (1990) Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. *Nature*, **346**, 284–287.

Hanson, D.D., Hamilton, D.A., Travis, J.L., Bashe, D.M. and Mascarenhas, J.P. (1989) Characterization of a pollen-specific cDNA clone from *Zea mays* and its expression. *Plant Cell*, **1**, 173–179.

Harlow, E. and Lane, D. (1988) *Antibodies. A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Heslop-Harrison, J. (1987) Pollen germination and pollen-tube growth. In *International Review of Cytology.* Volume 107, Pollen: Cytology and Development (Giles, K.L. and Prakash, J., eds). London: Academic Press, pp. 1–78.

Heslop-Harrison, J., Heslop-Harrison, Y. and Shivanna, K.R. (1984) The evaluation of pollen quality, and further appraisal of the fluorochromatic (FCR) test procedure. *Theor. Appl. Genet.* **67**, 367–375.

Itoh, K., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) Expression, glycosylation, and intracellular distribution of human β-galactosidase in recombinant baculovirus-infected *Spodoptera frugiperda* cells. *Biochem. Biophys. Res. Commun.* **167**, 746–753.

Jahnen, W., Lush, W.M. and Clarke, A.E. (1989) Inhibition of *in vitro* pollen tube growth by isolated S-glycoproteins of *Nicotiana alata*. *Plant Cell*, **1**, 501–510.

Jarvis, D.L. and Summers, M.D. (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell Biol.* **9**, 214–223.

Jofuku, K.D. and Goldberg, R.B. (1989) Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. *Plant Cell*, **1**, 1079–1093.

Kho, Y.O. and Baer, J. (1968) Observing pollen tubes by means of fluorescence. *Euphytica*, **17**, 298–302.

Kirchhoff, C., Habben, I., Ivell, R. and Krull, N. (1991) A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors. *Biol. Reprod.* **45**, 350–357.

Knox, R.B. and Heslop-Harrison, J. (1970) Pollen-wall proteins: Localization and enzymic activity. *J. Cell. Sci.* **6**, 1–27.

Knox, R.B., Heslop-Harrison, J. and Heslop-Harrison, Y. (1975) Pollen-wall proteins: localization and characterization of gametophytic and sporophytic fractions. In *The Biology of Male Gamete* (Duckett, J.G. and Racey, P.A., eds). *Biol. J. Linn. Soc.* Suppl. 1, Vol. VII, pp. 177–187.

Kortt, A.A., Strike, P.M. and De Jersey, J. (1989) Amino acid sequence of a crystalline seed albumin (winged bean albumin-1) from *Psophocarpus tetragonolobus* (L.) DC. Sequence similarity with Kunitz-type seed inhibitors and 7S storage globulins. *Eur. J. Biochem.* **181**, 403–408.

Kortt, A.A., Burns, J.E. and Strike, P.M. (1990) Amino acid sequence and disulfide bridges of affinity purified Kunitz-type chymotrypsin inhibitor from winged bean seed (*Psophocarpus tetragonolobus* (L.) DC). *Biochem. Int.* **22**, 543–552.

van der Krol, A.R., Mur, L.A., de Lange, P., Gerats, A.G.M., Mol, J.N.M. and Stuitje, A.R. (1990a) Antisense chalcone synthase in petunia: Visualization of variable transgene expression. *Mol. Gen. Genet.* **220**, 204–212.

van der Krol, A.R., Mur, L.A., de Lange, P., Mol, J.N.M. and Stuitje, A.R. (1990b) Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. *Plant Mol. Biol.* **14**, 457–466.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved

method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16–20.

Mascarenhas, J.P. (1993) Molecular mechanisms of pollen tube growth and differentiation. *Plant Cell*, **5**, 1303–1314.

McCormick, S. (1991a) Transformation of tomato with *Agrobacterium tumefaciens*. In *Plant Tissue Culture Manual: Fundamentals and Applications*, Volume B6 (Lindsey, K., ed.). Dordrecht: Kluwer, pp. 1–9.

McCormick, S. (1991b) Molecular analysis of male gametogenesis in plants. *Trends Genet.* **7**, 298–303.

McCormick, S. (1993) Male gametophyte development. *Plant Cell*, **5**, 1265–1275.

McCormick, S., Twell, D., Vancanneyt, G. and Yamaguchi, J. (1991a) Molecular analysis of gene regulation and function during male gametophyte development. In *Molecular Biology of Plant Development, SEB Symposium XLV* (Jenkins, G.I. amd Schuch, W., eds). Cambridge: Company of Biologists, pp. 229–244.

McCormick, S., Yamaguchi, J. and Twell, D. (1991b) Deletion analysis of pollen-expressed promoters. *In Vitro Cell Devel. Biol.* **27P**, 15–20.

Mo, Y., Nagel, C. and Taylor, L.P. (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc. Natl Acad. Sci. USA*, **89**, 7213–7217.

Obermeyer, G. and Kolb, H.-A. (1993) K^+ channels in the plasma membrane of lily pollen protoplasts. *Bot. Acta*, **106**, 26–31.

Obermeyer, G., Lützelschwab, M., Heumann, H.-G. and Weisenseel, M.H. (1992) Immunolocalization of H^+-ATPases in the plasma membrane of pollen grains and pollen tubes of *Lilium longiflorum*. *Protoplasma*, **171**, 55–63.

Oeller, P.W., Wong, L.-M., Taylor, L.P., Pike, D.A. and Theologis, A. (1991) Reversible inhibition of tomato fruit senescence by antisense RNA. *Science*, **254**, 437–439.

O'Reilly, D.R., Miller, L.K. and Lucknow, V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. New York: W.H. Freeman.

Palomares, R., Herrmann, R.G. and Oelmuller, R. (1993) Antisense RNA for components associated with the oxygen-evolving complex and the Rieske iron/sulfur protein of the tobacco thylakoid membrane suppresses accumulation of mRNA, but not of protein. *Planta*, **190**, 305–312.

Polowick, P.L. and Sawhney, V.K. (1993) An ultrastructural study of pollen development in tomato (*Lycopersicon esculentum*) II. Pollen maturation. *Can. J. Bot.* **71**, 1048–1055.

Preuss, D., Lemieux, B., Yen, G. and Davis, R.W. (1993) A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization. *Genes Devel.* **7**, 974–985.

Rao, K.S. and Kristen, U. (1990) The influence of the detergent Triton X-100 on the growth and ultrastructure of tobacco pollen tubes. *Can. J. Bot.* **68**, 1131–1138.

Regan, S.M. and Moffatt, B.A. (1990) Cytochemical analysis of pollen development in wild-type *Arabidopsis* and a male-sterile mutant. *Plant Cell*, **2**, 877–889.

Richardson, M. (1977) The proteinase inhibitors of plants and micro-organisms. *Phytochemistry*, **16**, 159–169.

Rick, C.M. and Dempsey, W.H. (1969) Position of the stigma in relation to fruit setting of the tomato. *Bot. Gaz.* **130**, 180–186.

Riesmeier, J.W., Flügge, U.-I., Schulz, B., Heineke, D., Heldt, H-W., Willmitzer, L. and Frommer, W.B. (1993) Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants. *Proc. Natl Acad. Sci. USA*, **90**, 6160–6164.

Ritonja, A., Kopitar, M., Jerala, R. and Turk, V. (1989) Primary structure of a new cysteine proteinase inhibitor from pig leucocytes. *FEBS Lett.* **255**, 211–214.

Robert, L.S., Donaldson, P.A., Ladaique, C., Altosaar, I., Arnison, P.G. and Fabijanski, S.F. (1989) Antisense RNA inhibition of β-glucuronidase gene expression in transgenic tobacco plants. *Plant Mol. Biol.* **13**, 399–409.

Rodermel, S.R., Abbott, M.S. and Bogorad, L. (1988) Nuclear–organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell*, **55**, 673–681.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schlüpmann, H., Bacic, A. and Read, S.M. (1993) A novel callose synthase from pollen tubes of *Nicotiana*. *Planta*, **191**, 470–481.

Shivanna, K.R., Heslop-Harrison, J. and Heslop-Harrison, Y. (1981) Heterostyly in *Primula*. 2. Sites of pollen inhibition, and effects of pistil constituents on compatible and incompatible pollen tube growth. *Protoplasma*, **107**, 319–337.

Shivanna, K.R., Heslop-Harrison, J. and Heslop-Harrison, Y. (1983) Heterostyly in *Primula*. 3. Pollen water economy: a factor in the intramorph-incompatibility response. *Protoplasma*, **117**, 175–184.

Spindler, K.R., Rosser, D.S.E. and Berk, A.J. (1984) Analysis of Adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. *J. Virol.* **49**, 132–141.

Steer, M.W. and Steer, J.M. (1989) Pollen tube tip growth. *New Phytol.* **111**, 323–358.

Stockhaus, J., Hofer, M., Renger, G., Westhof, P., Wydrzyski, T. and Willmitzer, L. (1990) Anti-sense RNA efficiently inhibits formation of the 10 kd polypeptide of photosystem II in transgenic potato plants: analysis of the role of the 10 kd protein. *EMBO J.* **9**, 3013–3021.

Temple, S.J., Knight, T.J., Unkefer, P.J. and Sengupta-Gopalan, C. (1993) Modulation of glutamine synthetase expression in tobacco by the introduction of an alfalfa glutamine synthetase gene in sense and antisense orientation: molecular and biochemical analysis. *Mol. Gen. Genet.* **236**, 315–325.

Tieman, D.M., Harriman, R.W., Ramamohan, G. and Handa, A.K. (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. *Plant Cell*, **4**, 667–679.

Twell, D. (1992) Use of a nuclear-targeted β-glucuronidase fusion protein to demonstrate vegetative cell-specific gene expression in developing pollen. *Plant J.* **2**, 887–892.

Twell, D., Klein, T.M., Fromm, M.E. and McCormick, S. (1989a) Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. *Plant Physiol.* **91**, 1270–1274.

Twell, D., Wing, R., Yamaguchi, J. and McCormick, S. (1989b) Isolation and expression of an anther-specific gene from tomato. *Mol. Gen. Genet.* **217**, 240–245.

Twell, D., Yamaguchi, J. and McCormick, S. (1990) Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. *Development*, **109**, 705–713.

Twell, D., Yamaguchi, J., Wing, R.A., Ushiba, J. and McCormick, S. (1991) Promoter analysis of genes that are

coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Devel.* **5**, 496–507.

Vernet, T., Tessier, D.C., Richardson, C., Laliberté, F., Khouri, H.E., Bell, A.W., Storer, A.C. and Thomas, D.Y. (1990) Secretion of the papain precursor from insect cells. Requirement for N-glycosylation of the pro-region. *J. Biol. Chem.* **265**, 16661–16666.

Villalba, M., Batanero, E., Lopez-Otin, C., Sanchez, L.M., Monsalve, R.I., Gonzalez de La Pena, M.A., Lahoz, C. and Rodriguez, R. (1993) The amino acid sequence of *Ole e I*, the major allergen from olive tree (*Olea europaea*) pollen. *Eur. J. Biochem.* **276**, 863–869.

Weisenseel, M.H. and Jaffe, L.F. (1976) The major growth current through lily pollen tubes enters as K^+ and leaves as H^+. *Planta*, **133**, 1–7.

Willlams, E.G., Knox, R.B. and Rouse, J.L. (1982) Pollination sub-systems distinguished by pollen tube arrest after incompatible interspecific crosses in *Rhododendron* (Ericaceae). *J. Cell Sci.* **53**, 255–277.

Zhang, H., Scheirer, D.C., Fowle, W.H. and Goodman, H.M. (1992) Expression of antisense or sense RNA of an ankyrin repeat-containing gene blocks chloroplast differentiation in *Arabidopsis*. *Plant Cell*, **4**, 1575–1588.

Zou, J., Zhan, X., Wu, H., Wang, H. and Cheung, A.Y. (1994) Characterization of a rice pollen-specific gene and its expression. *Am. J. Bot.* **81**, 552–561.